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Biochemistry

Nitric oxide signaling to iron-regulatory protein: Direct control of ferritin mRNA translation and transferrin receptor mRNA stability in transfected fibroblasts

KOSTAS PANTOPOULOS AND MATTHIAS W. HENTZE*

Gene Expression Programme, European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

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ABSTRACT **Iron-regulatory protein (IRP) is a master regulator of cellular iron homeostasis. Expression of several genes involved in iron uptake, storage, and utilization is regulated by binding of IRP to iron-responsive elements (IREs), structural motifs within the untranslated regions of their mRNAs. IRP-binding to IREs is controlled by cellular iron availability. Recent work revealed that nitric oxide (NO) can mimic the effect of iron chelation on IRP and on ferritin mRNA translation, whereas the stabilization of transferrin receptor mRNA following NO-mediated IRP activation could not be observed in γ-interferon/lipopolysaccharide-stimulated murine macrophages. In this study, we establish the function of NO as a signaling molecule to IRP and as a regulator of mRNA translation and stabilization. Fibroblasts with undetectable levels of endogenous NO synthase activity were stably transfected with a cDNA encoding murine macrophage inducible NO synthase. Synthesis of NO activates IRE binding, which in turn represses ferritin mRNA translation and stabilizes transferrin receptor mRNA against targeted degradation. Furthermore, iron starvation and NO release are shown to be independent signals to IRP. The post-transcriptional control of iron metabolism is thus intimately connected with the NO pathways.**

Iron-responsive elements (IREs) confer posttranscriptional iron-dependent regulation on several mRNAs related to iron metabolism. The high-affinity interaction of IREs with a cytoplasmic protein, iron-regulatory protein (IRP), provides a versatile mechanism for the coordinated regulation of the fate of these mRNAs: an IRE/IRP complex in the 5′ untranslated region (UTR) of ferritin and erythroid 5-aminolevulinate synthase (eALAS) mRNAs inhibits their translation by preventing the stable association of the small ribosomal subunit with these messages (1). Interaction of IRP with five IREs in the 3′ UTR of transferrin receptor (TfR) mRNA stabilizes this transcript against targeted endonucleolytic degradation (2). Thus, cellular iron uptake (TfR), storage (ferritin), and utilization (eALAS) are controlled posttranscriptionally by IRE/IRP interactions (for reviews, see refs. 3 and 4). Changes in cellular iron levels alter the structure and function of IRP. In iron-loaded cells, IRE binding is prevented by a 4Fe-4S cluster in the core region of the protein (Fe-S IRP), whereas the clusterless apo-IRP predominates in iron-starved cells and binds tightly to IREs (5, 6). As 4Fe-4S IRP, the protein displays aconitase activity, revealing its bifunctional character and underscoring its relationship with the mitochondrial aconitase of the Krebs cycle (7–10). It is not known how cellular iron levels affect assembly/disassembly of the Fe-S cluster.

The functional switch between the IRE-binding and the aconitase activity of IRP can also be operated by nitric oxide (NO) (reviewed in ref. 11). *In vitro*, treatment of recombinant IRP with NO gas increases IRE binding and abolishes the aconitase activity (12). In murine peritoneal macrophages and in the macrophage cell lines RAW 264.7 and J774.A1, γ-interferon (IFN-γ) and lipopolysaccharide (LPS) induce NO synthase (NOS). The release of NO correlates with activation of IRE binding and aconitase inactivation, followed by repression of ferritin biosynthesis (12, 13). These responses to NO resemble the effects of iron chelation, and suppression of NO synthesis in stimulated macrophages by the arginine analog N^G-monomethyl-L-arginine (NMMA) reduces the IRE-binding activity below the level in untreated control cells (12, 13). Yet no effect of NO-mediated activation of IRE-binding on TfR mRNA stability is observed (12). These results and the finding that iron starvation induces transcription of inducible NOS (iNOS) mRNA in J774.A1 cells (14) raise questions concerning the regulatory connections between iron metabolism and NO. Are the responses of IRP to iron starvation and NO coupled or independent? Does NO activation of IRP (as opposed to the activation by iron starvation) only repress ferritin translation but fail to stabilize TfR mRNA? Do pharmacological agents used to stimulate NO synthesis provoke additional cellular responses that are required to facilitate the effect of NO on IRP and its target mRNAs?

To address these questions in a well-defined system, murine B6 fibroblasts were stably transfected to express murine macrophage iNOS. NO release from iNOS suffices to directly control both ferritin mRNA translation and TfR mRNA stability. We present evidence that NO elicits the activation of IRE binding by triggering disassembly of the Fe-S cluster in IRP and that activation of IRE binding by iron starvation and NO are independent, converging pathways.

MATERIALS AND METHODS

Expression of iNOS in B6 Fibroblasts. Murine macrophage iNOS cDNA was excised with *Hin*cII/*Ssp* I from piNOSL3 (15) and subcloned into the *Sma* I site of the simian virus 40 promoter-driven expression vector pSG5 (16), containing a polylinker sequence from pMS56 (10). pSG5iNOS (15 μg) and pHSV106 (0.25 μg) [containing the herpes simplex virus thymidine kinase gene (*tk*); BRL] were cotransfected by the calcium phosphate coprecipitation method into B6 fibroblasts (17). Stable transformants (B6.NOS) were selected and maintained in supplemented Dulbecco's modified Eagle's medium (DMEM) containing hypoxanthine/aminopterin/thymidine (HAT). A colony of B6 fibroblasts cotransfected with *tk* and a luciferase cDNA (instead of iNOS cDNA) which does not display any NOS activity was used as a control cell line

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Abbreviations: IRE, iron-responsive element; IRP, iron-regulatory protein; TfR, transferrin receptor; NOS, NO synthase; iNOS, inducible NOS; NMMA, N^G-monomethyl-L-arginine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; IFN, interferon; hGH, human growth hormone; 2-ME, 2-mercaptoethanol; UTR, untranslated region.

*To whom reprint requests should be addressed.

(B6.ctrl) (these cells have possibly incorporated only *tk*, since they grow in HAT medium but do not express luciferase mRNA or enzyme activity).

Transient Transfection of B6.NOS and B6.ctrl Cells. The synthetic 250-nt minimal regulatory element of the TfR gene was excised from the plasmid TRS-1 (18) with *Sac* I/*Sal* I and inserted into the respective sites of pGEM-luc (Promega). The lucTRS-1 cassette was cut with *Bam*HI and subcloned into pSG5, giving pSG5luc.TRS-1. pSG5luc.TRS-3 was constructed similarly from TRS-3 (18) and pGEM-luc. pSG5hGH.TRS-1 was generated by replacement of the luciferase coding region (*Eco*NI linearization followed by blunt-end formation with Klenow DNA polymerase and then by *Eco*RI digestion) with a PCR-amplified cDNA encoding the 22-kDa human growth hormone (hGH) variant (19). pSG5hGH.TRS-1 (5 μg) and pSG5luc.TRS-3 (1 μg) were cotransfected by lipofection (Boehringer Mannheim) into B6.NOS and B6.ctrl cells (20). Luciferase assays were as described (21).

Cell Treatments. B6.NOS and B6.ctrl cells were pretreated with 5 mM sodium butyrate (to augment transcription from the transfected NOS minigene) and 5 μM ferric ammonium citrate (to prevent cellular iron deprivation) for 8 hr and then treated for the indicated times with 400 μM NMMA, 10 mM arginine, 100 μM hemin, or 100 μM desferrioxamine in fresh medium (containing 5 mM butyrate and 5 μM ferric ammonium citrate). RAW 264.7 and J774.A1 murine macrophages were grown in supplemented DMEM and stimulated for 8 hr with IFN-γ (50 units/ml) plus LPS (10 μg/ml) from *Escherichia coli*. Subsequent treatments were in the presence of IFN-γ/LPS.

Nitrite Assay. A 0.25-ml sample of culture medium was incubated with 0.75 ml of Griess reagent (Merck) for 10 min at room temperature, and A_{543} was measured. NOS enzymatic assays of cell extracts were as described (22).

Gel Retardation Assays. A ^{32}P-labeled IRE probe was transcribed *in vitro* from the plasmid I-12.CAT (10) (linearized with *Xba* I) by T7 RNA polymerase and purified by gel electrophoresis (23). The transcript had the sequence 5′-GGGCGAAUUCGAGCUCGGUACCCGGGGAUCCU-GCUUCAACAGUGCUUGGACGGAUCCU-3′, where the unpaired C residue and the loop of the IRE are underlined. Detergent extracts of cells were prepared (24), and gel retardation assays were performed (25).

Metabolic Labeling and Immunoprecipitation. Cells were metabolically labeled for 2 hr with [^{35}S]methionine (50 μCi/ml; 1 μCi = 37 kBq) in methionine-free medium. Hemin, desferrioxamine, NMMA, or arginine treatment was continued during metabolic labeling. Quantitative immunoprecipitation from equal amounts of trichloroacetic acid-insoluble radioactivity was performed with polyclonal ferritin antibodies (Boehringer Mannheim) and U1A antiserum (kindly provided by Iain Mattaj, European Molecular Biology Laboratory, Heidelberg) and followed by SDS/PAGE and autoradiography (26). Quantitative analysis was by phosphorimaging.

Northern Blot Analysis. Cells were lysed with RNA-clean solution (AGS, Heidelberg) and RNA was prepared according to the manufacturer's recommendations. Samples (10 μg) of RNA were electrophoretically resolved in denaturing agarose gels and electrotransferred onto nylon membranes. The RNA was UV crosslinked to the membrane and hybridized to radiolabeled probes.

RESULTS

NO Directly Activates IRE Binding by IRP *in Vivo*. NO synthesis can be elicited, depending on cell type, by cytokines such as IFN-γ, by stimulation of calcium influx, or by addition of NOS cofactors such as tetrahydrobiopterin (reviewed in refs. 27–29). In addition, these signals commonly trigger a multitude of other cellular responses. The ability of L-arginine analogs, such as NMMA or N^G-nitro-L-arginine, to competitively inhibit NO synthesis from L-arginine is commonly used to distinguish between NO effects and other cellular responses. While this approach helps to identify whether NO synthesis is required, the contribution of additional cellular responses to modulation of the parameters under investigation has to be considered.

We generated a fibroblast cell line (B6.NOS) which stably expresses murine macrophage iNOS mRNA from a viral promoter. B6.NOS cells constitutively synthesize NO (Fig. 1*A*) and thus provide an experimental system for NO signaling to IRP, avoiding possible indirect effects of cytokine stimulation on cellular metabolism. In B6.NOS cells, IRE binding by IRP is highly activated (Fig. 1*A*): (*i*) Inhibition of NO synthesis by

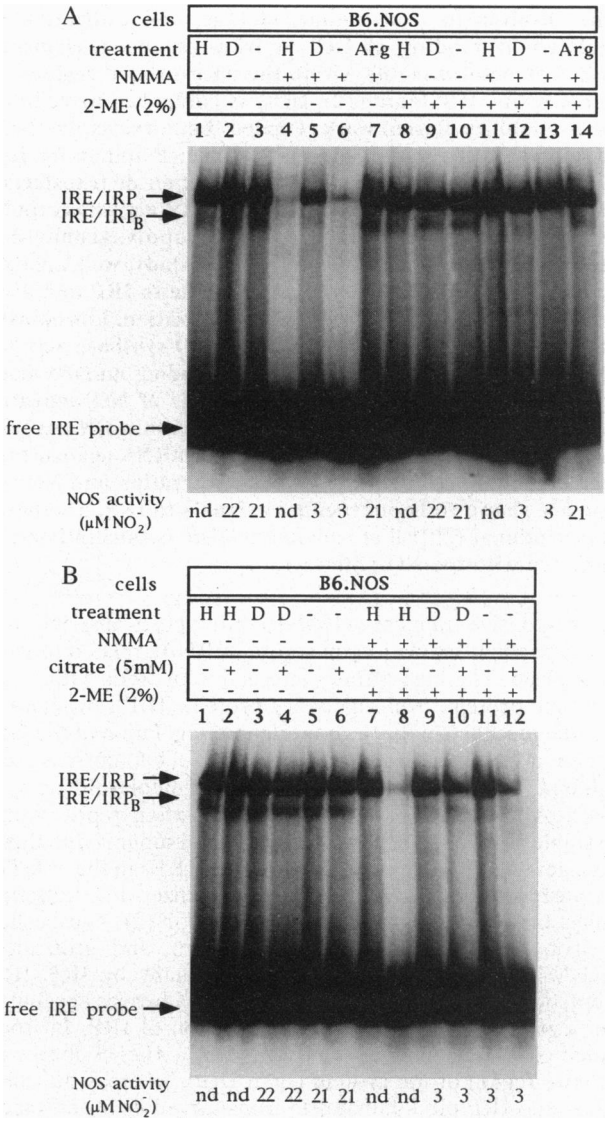

FIG. 1. Effect of NO on the IRE-binding activity of IRP in B6.NOS fibroblasts. Cytoplasmic extracts (25 μg) of B6.NOS cells were used for gel retardation assays with 25,000 cpm of radiolabeled IRE probe. (*A*) 2-Mercaptoethanol [2-ME (2%)] was added prior to addition of probe to samples in lanes 8–14. Where indicated, cells were treated for 12 hr with NMMA and/or arginine, and hemin (H) or desferrioxamine (D) was added subsequently for another 4 hr. Nitrite levels in the culture medium (*Lower*) were assessed prior to cell harvest and reflect the NOS activity of the cells (nd, not determined, due to interference of hemin with the nitrite assay). (*B*) Citrate (5 mM) and/or 2-ME (2%) was added, where indicated, prior to the probe. Positions of excess free probe and specific RNA/protein complexes are indicated by arrows.

treatment with NMMA strongly diminished IRE binding, which was restored by an excess of arginine (lanes 1 and 3 vs. lanes 4, 6, and 7). (*ii*) IRE-binding-activity induction by NO was observed even in iron-replete cells pretreated with hemin (lane 1), which normally strongly diminishes IRE binding. (*iii*) NO-induced IRE binding by IRP was only marginally stimulated by addition of 2% 2-ME to the extract (lanes 1–3 vs. lanes 8–10), whereas a strong *in vitro* induction by 2-ME was observed in extracts from cells where NO synthesis was blocked (lanes 4 and 6 vs. lanes 11 and 13). (*iv*) IRE binding was not appreciably stimulated in B6.NOS cells by addition of the iron chelator desferrioxamine (lane 2 vs. lanes 1 and 3). NO also induces the IRE-binding activity of IRP_B, a second specific IRE-binding protein so far identified only in rodent cells (30). The IRE-binding activity of IRP_B is iron- and NO-regulated in the same fashion as IRP, but unlike IRP, was not induced *in vitro* by 2% 2-ME (lanes 4–6 vs. lanes 11–13). We conclude that NO can directly activate IRE binding by IRP and IRP_B without treatment of cells with agents such as IFN-γ/LPS or tetrahydrobiopterin.

Is NO-Activated IRP Also an Apoprotein? NO induces the IRE-binding activity and inhibits the aconitase activity of IRP (12). Inactivation of the mitochondrial aconitase in tumor cells by macrophages stimulated to produce NO correlates with the removal of a labile iron atom from the 4Fe-4S cluster (31). Does activation of IRE binding of IRP also involve modifications of its 4Fe-4S cluster? Several lines of evidence indicate that the apoprotein is the relevant IRE-binding form of IRP in iron-deprived cells, while Fe-S IRP predominates in iron-loaded cells (5, 6, 9). The aconitase substrates citrate and *cis*-aconitate can tightly interact with the 4Fe-4S or 3Fe-4S forms of IRP, even when IRP is reversibly activated for IRE binding with 2% 2-ME *in vitro*. When bound to an Fe-S cluster, citrate interferes with 2-ME-activated IRE binding. In contrast, aconitase substrates do not affect IRE binding by apo-IRP (6). As expected, citrate (5 mM) competed for IRE binding in 2-ME-treated extracts from iron-loaded B6.NOS cells, when NO biosynthesis was blocked by NMMA (Fig. 1*B*, lanes 7 and 8); had only a small effect on non-iron-perturbed NMMA-treated cells (lanes 11 and 12); and did not affect IRE binding in extracts from iron-depleted cells (lanes 9 and 10). Citrate had no effect on IRE binding by IRP and IRP_B in extracts from B6.NOS cells, whether previously iron-loaded with hemin (lanes 1 and 2), iron-depleted with desferrioxamine (lanes 3 and 4), or non-iron-perturbed (lanes 5 and 6). These results suggest that in B6.NOS cells, NO acts by causing the disassembly of the 4Fe-4S cluster beyond the 3Fe-4S stage. This could be a consequence of a direct reaction of NO with the Fe-S cluster, a response to NO-induced second messengers that act on IRP; alternatively, NO could exert its function by reducing the level of iron available for cluster formation and maintenance.

Iron and NO Signaling to IRP Originate as Independent Pathways. Fig. 1 reveals a strong resemblance between the effects of iron chelation and of NO on IRP. Likewise, activation of IRE binding by NO in the macrophage cell lines RAW 264.7 and J774.A1 resembles the effects of iron depletion (12, 13). Iron depletion induces iNOS transcription in J774.A1 cells, whereas iron administration has a negative effect on iNOS expression (14). These results raise the question of whether NO synthesis is an essential requirement for the formation of the IRE-binding form of IRP under conventional conditions of iron deprivation, possibly even in cells where NOS activity is undetectable by methods commonly employed. The fibroblast cell line B6 (parent to the B6.NOS cells) and B6.ctrl cells were investigated to evaluate this hypothesis. B6 cells display no endogenous NOS activity, even after treatment with IFN-γ/LPS, as judged by measurement of nitrite in the culture supernatant or by direct NOS assay of cell extracts (data not shown). If an iron-regulated NOS activity escaping

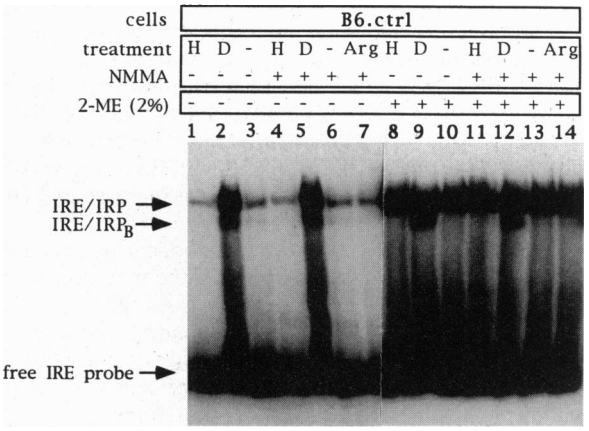

FIG. 2. NMMA does not affect the IRP response to iron starvation in B6.ctrl fibroblasts. B6.ctrl cells were treated and cytoplasmic extracts analyzed by gel retardation assay as described in Fig. 1.

detection by standard assays was involved in conventional IRP regulation by iron, the increase in IRE binding following cellular iron starvation should be negatively affected by the inhibitory NOS substrate analog NMMA. While hemin abolished and desferrioxamine induced IRE-binding in B6.ctrl cells (Fig. 2, lanes 1–3), treatment with NMMA (and arginine) failed to influence IRE binding irrespective of the cellular iron status (lanes 4–7). Thus, iron chelation activates IRE-binding under conditions which block the NO effect on IRP in B6.NOS cells, indicating that iron signaling to IRP can occur independently from NO, at least in B6.ctrl cells.

Regulation of Translation and mRNA Stability by NO. As would be expected from previous results with IFN-γ/LPS-induced J774.A1 cells, ferritin biosynthesis was repressed by activated IRP in B6.NOS cells (Fig. 3 *Upper*). This repression was particularly evident in iron-loaded cells, where the rate of ferritin synthesis was normally very high (lane 1 vs. lanes 4, 7, and 10). The expression of the spliceosomal protein U1A (which served as an internal control) was unaffected by NO or NMMA. Northern blot analysis (Fig. 3 *Lower*) showed that the inhibition of ferritin biosynthesis was translational and not

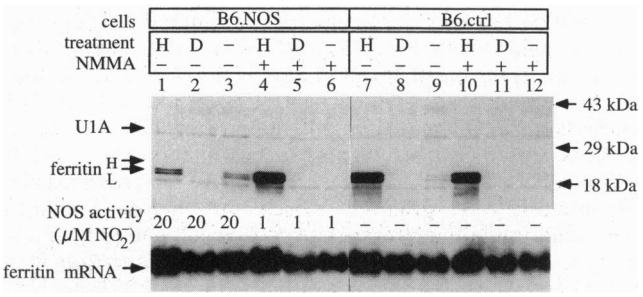

FIG. 3. NO-mediated repression of ferritin mRNA translation. B6.NOS and B6.ctrl fibroblasts were treated with NMMA, as indicated. After 12 hr, hemin (H) or desferrioxamine (D) was added and incubation was continued for 4 hr. (*Upper*). Cells were metabolically labeled with [^{35}S]methionine. Half of the cells was lysed and quantitative immunoprecipitation with ferritin and U1A antibodies was performed. Immunoprecipitated endogenous ferritin and U1A (internal control) from B6.NOS (lanes 1–6) and B6.ctrl (lanes 7–12) cells were analyzed by SDS/PAGE. Heavy (H) and light (L) chains of ferritin are indicated. Nitrite in the culture medium was determined prior to addition of hemin and desferrioxamine (−, no detectable nitrite). (*Lower*). RNA was extracted from the other half of the cells. RNA (10 μg per lane) was resolved in a denaturing formaldehyde/agarose gel and electrotransferred onto a nylon filter, which was then hybridized with a radiolabeled human ferritin heavy-chain cDNA probe.

associated with an NO-induced decrease in ferritin mRNA (which was slightly increased in hemin-treated cells due to transcriptional induction by hemin). Consistent with the conclusions from Fig. 2, repression of ferritin synthesis by iron chelation in B6.ctrl cells was unaffected by NMMA (Fig. 3 *Upper*, lanes 8 and 11). The small increase in ferritin biosynthesis in untreated B6.NOS compared with B6.ctrl cells (lanes 3 and 9) was most likely related to a minor increase in ferritin mRNA (Fig. 3 *Lower*, lanes 3 and 9).

IRP also controls cellular iron metabolism by regulating TfR mRNA stability (2). We thus tested the macrophage cell lines J774.A1 and RAW 264.7 for effects of NO on the regulation of TfR mRNA stability. Iron loading reduced TfR mRNA levels, whereas iron depletion protected the TfR mRNA (Fig. 4*A*, lanes 1–3). Stimulation of cells with IFN-γ/LPS induced iNOS mRNA transcription followed by NOS biosynthesis and NO production, which was inhibited by NMMA and restored by an excess of arginine (lanes 4–6). Surprisingly, NO release was accompanied by a decrease rather than an increase in TfR mRNA (lanes 4–6), whereas the internal control, GAPDH mRNA, remained unchanged. The same results were obtained with J774.A1 cells (data not shown). Thus, TfR mRNA stabilization was not observed following NO synthesis in macrophage cell lines, suggesting that the regulatory region of TfR mRNA is unresponsive to the NO effect on IRP or that macrophage stimulation may cause additional effects on TfR expression.

To distinguish between these possibilities, we examined TfR expression in B6.NOS cells. B6.NOS and B6.ctrl cells were iron-manipulated in the absence or presence of NMMA, and RNA was extracted for Northern analysis. NO production in B6.NOS cells led to a 6-fold increase in TfR mRNA compared with B6.ctrl cells (Fig. 4*B*, lanes 3 and 10). TfR mRNA was further induced by desferrioxamine (compare lanes 2, 3, and 9). TfR mRNA was also increased in hemin-treated B6.NOS cells compared with hemin-treated B6.ctrl cells (lanes 1 and 8). These responses were NO-dependent, as they were inhibited by NMMA and restored by excess arginine (lanes 4–7). NMMA and arginine had no effect on TfR mRNA levels in B6.ctrl cells, and GAPDH mRNA was unaffected in B6.NOS and B6.ctrl cells. Hybridization with an iNOS probe demonstrated the steady-state iNOS mRNA expression in the B6.NOS cells. Thus, TfR mRNA expression was up-regulated by NO. A hybrid hGH reporter mRNA bearing a minimal 3′ UTR regulatory region from the TfR mRNA was also stabilized in transiently transfected B6.NOS cells (Fig. 4*C*), showing that the NO effect was mediated by IRE/IRP interactions. We conclude that NO regulates the stability of TfR mRNA in B6.NOS cells. These results also suggest that TfR expression is controlled at multiple levels in murine macrophages in response to IFN-γ, perhaps by a nuclear mechanism similar to that found in the human amniotic WISH cell line (32). Interestingly, activation of IRE binding by iron starvation in a murine T-cell line also does not suffice to increase TfR mRNA, but the presence of interleukin 2 is required in addition to desferrioxamine (33).

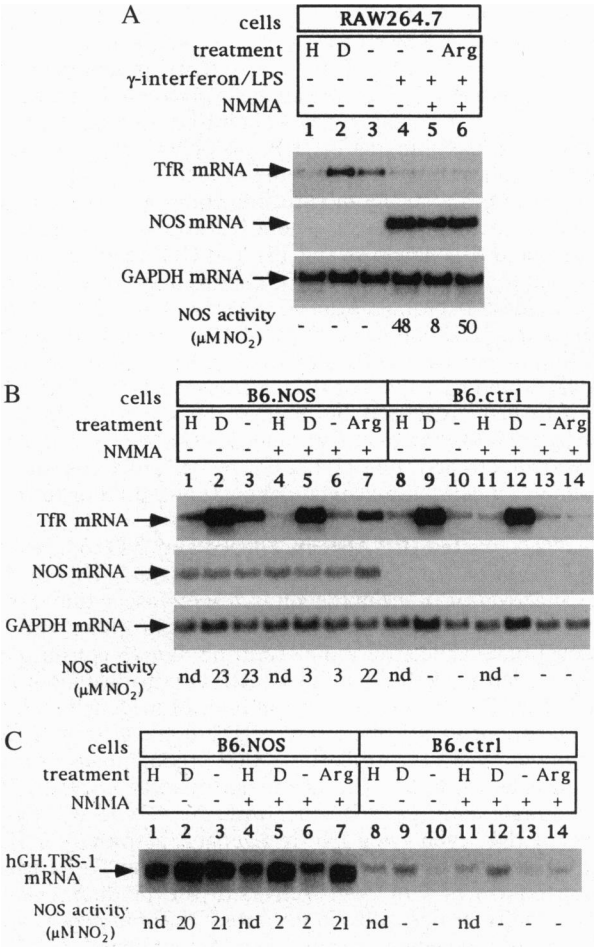

FIG. 4. (*A* and *B*) Effect of NO on TfR mRNA levels in RAW264.7 macrophages (*A*) and B6.NOS and B6.ctrl cells (*B*). Cells were treated for 12 hr with hemin (H) or desferrioxamine (D) or stimulated with IFN-γ/LPS and treated with arginine and/or NMMA. Total RNA was extracted, and samples (10 μg) were resolved in formaldehyde/agarose gels and electrotransferred onto nylon filters. The filters were hybridized with a murine TfR cDNA probe, then with a murine iNOS cDNA, and finally with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. (*C*) B6.NOS and B6.ctrl cells were transiently transfected with 5 μg of pSG5hGH.TRS-1 and 1 μg of pSG5luc.TRS-3 (internal control, non-iron-regulated) and treated with hemin, desferrioxamine, or arginine and/or NMMA for 12 hr. RNA was extracted and analyzed as described above. A small fraction of the transfected cells was assayed for luciferase activity. Transfection efficiencies were highly similar within the groups of transfected B6.NOS and B6.ctrl cells but were ≈10-fold higher in the B6.NOS transfectants than in the B6.ctrl transfectants (data not shown). Nitrite in the culture medium was assessed prior to cell harvest (−, no detectable nitrite; nd, not determined, due to the interference of hemin with the nitrite assay).

DISCUSSION

To our knowledge, the IRE/IRP system represents the first example of a direct involvement of NO in regulating gene expression. The recent discovery of NO as a signaling molecule in various biochemical processes, the identification of iron-containing proteins as its main physiological targets, and the study of its chemical reactivity toward iron suggest ample reasons for a regulatory role of NO in cellular iron metabolism (11). The Fe-S cluster of IRP has been considered as the likely target of NO (11–13). In accord with this hypothesis, both its aconitase and IRE-binding activities were shown to be affected by NO *in vitro* (12) and in murine macrophages stimulated to synthesize NO (12, 13).

As does iron chelation by desferrioxamine, NO biosynthesis activates IRE-binding by IRP. In B6.NOS cells treated with desferrioxamine, a stronger repression of ferritin mRNA translation (Fig. 3) and stabilization of TfR mRNA (Fig. 4) were observed than in untreated cells. The induction of IRE binding by iron starvation may result from a conformational change between Fe-S IRP and apo-IRP. Our results suggest that NO activation of IRE binding also operates by cluster disassembly, as citrate does not affect IRE binding in extracts from B6.NOS cells (Fig. 1*B*), a property of apo-IRP which is not shared by 3Fe-4S or 4Fe-4S forms of IRP (5, 6). Cellular NO synthesis mimics the effects of iron depletion on IRP in

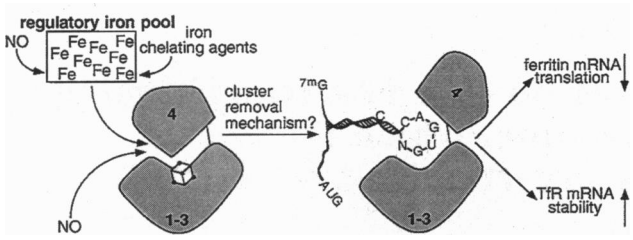

FIG. 5. Possible mechanisms for the activation of IRE binding by NO. IRP is schematically depicted as a four-domain Fe-S protein, with domain 4 linked to domains 1–3 by a hinge linker. NO may interact directly with IRP (possibly the cubane 4Fe-4S cluster), stimulate second messengers, and/or modulate the regulatory iron pool. In either case, a conformational change of the protein may be induced by direct NO-mediated cluster disassembly and/or indirectly as a result of a decrease in iron (sequestration of chelatable iron by NO). The induction of IRE-binding activity represses ferritin mRNA translation and protects TfR mRNA from targeted degradation.

several respects. NO is not required for the cellular response to iron starvation (Fig. 2). However, iron starvation stimulates NOS activity in J774.A1 cells (14) and may thus potentiate its effect on IRP in these cells. Future work will have to define whether NO regulates IRP by reacting with its Fe-S cluster or by activating cluster disassembly indirectly as a consequence of modulation of the so called "regulatory iron pool" by formation of iron-nitrosyl complexes (Fig. 5). The conversion of 4Fe-4S IRP to the IRE-binding form by NO gas *in vitro* (12) argues for a direct effect, but it is noteworthy that this effect is incomplete. Likewise, the possible role of second messengers (such as superoxide anion) needs to be considered. However, the stimulation of IRE binding by the NO (and superoxide)-releasing agent SIN-1 is increased (rather than diminished) by the inclusion of superoxide dismutase (12), and treatment of cells for 1 hr with 500 μM paraquat (which releases superoxide) has no effect on the IRE-binding activity of IRP (unpublished data), arguing that superoxide does not serve as a second messenger to regulate IRP.

In summary, we have described a role of NO in directly regulating gene expression: NO synthesis causes repression of ferritin mRNA translation and stabilization of TfR mRNA against targeted degradation. Both responses result from the NO-mediated activation of the RNA-binding activity of IRP. This activation is most likely caused by apo-IRP formation. Thus, both iron starvation and NO synthesis trigger apo-IRP accumulation in cells, and the two signaling pathways originate independently. Since NO can diffuse from NO-producing into neighboring cells, it may also affect their iron metabolism. With the molecular basis of NO-mediated effects on IRP, ferritin, and TfR being established, the physiological and pathological consequences on the regulation of iron metabolism are likely to be unraveled further.

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